REMARKS

Claims 1-39 are pending in the instant application. Claims 1-32, 34, and 36-39 are rejected. Claims 33, 35 and 38 are withdrawn from consideration.

Objection to the claims

The Examiner correctly says that claim 1, step i) is believed to mean "0.5 to 5% D-xylose." Applicants herein correct the inadvertent typographical error.

Rejection under 35 USC 112, first paragraph

The Examiner rejects claim 20 as not enabled because the claim recites that the reaction is stopped by cooling to 0° C whereas the specification indicates that the reaction continues to -5°C. The Examiner is absolutely correct. The reaction does not stop at O°C, rather it slows down sufficiently to be able to continue with the fourth step of the process (extraction of the agliconic fragment from the substrate). Applicants herein change claim 20, changing the verb "stopped" to "slowed," to bring the claims in conformity with the teachings of the specification.

Rejection under 35 USC 103

1. *Reyes et al., U.S. Patent 5,994,092 in view of Ponpipom et al., U.S. Patent 4,228,274 and Crumpton et al., Biochem. J. 70(4):729 (1958)*

The Examiner rejects claims 1-4, 21-24, 27-32, 34, 36, 37 and 39 as unpatentable over Reyes et al., U.S. Patent 5,994,092 in view of Ponpiporn et al., U.S. Patent 4,228,274 and Crumpton et al., Biochem. J. 70(4):729 (1958). The Examiner admits that Reyes et al. do not teach crystallization of 4GPX, however, the Examiner says that crystallization is a common procedure for the purification of saccharides as taught by Ponpipom et al. and that one of ordinary skill in the art would recognize that recrystallization is a common process for isolating saccharides. Further, the Examiner says that the choice of solvent is a matter of routine optimization absent evidence of unexpectedly superior results.

As the Examiner knows, in order to establish a proper *prima facie* case of obviousness, the Examiner must establish that there is a suggestion or motivation to modify the references or

to combine the reference teachings; there must be a reasonable expectation of success; and the references or combination of references must teach or suggest all of the claim limitations (*see, e.g.,* MPEP § 2142). The teachings or suggestions to make the claimed combination and the reasonable expectation of success must both be found in the prior art and not based on applicant's disclosure (*In re Vaeck*, 947 F.2d 488, 20 USPQ2d 1438 (Fed. Cr. 1991)). The arguments advanced by the Examiner fail to meet all of these criteria.

Regarding methods for purifying carbohydrates

The present claims recite obtaining a product utilizable for evaluating intestinal lactase. In the process described two parts can be distinguished: an enzyme reaction and a subsequent purification of the reaction mixture. The reasons for the Examiner's rejection refer to the purification methods used as being well known and routine. Applicants respectfully submit that developing a method for purifying carbohydrate mixtures is neither easy nor routine for one skilled in the art, due to the characteristic of these molecules.

In effect, the chemistry of carbohydrates is very complex, since they are molecules which, amongst themselves, have a very similar structure (many have the same molecular fonnula; for example, lactose and sucrose both possess 12 carbons, 22 hydrogens and 11 oxygens) and with the same type of functional group (the hydroxyl group). These complexities in the chemistry of carbohydrates are well known and acknowledged by organic chemists of ordinary skill in the art. For example, the words of Y. Kishi may highlight the complexities of carbohydrate chemistry, referring specifically to the reaction of glycosylation in the synthesis of erythromycin:

> *"Glycosylation is a very old reaction but one of the hardest to achieve. We tried many old methods before we found a successful one. It was not quite new but there's a lot of small but important know-how to glycosylation. Any time you talk of sugars in organic synthesis, it means headaches"* (J. L. Fox. *Chem. Eng. News,* 1981,59,32.)

Small changes in the structure of carbohydrates (for example, two carbohydrates that differ in the esterochemistry of one of their chiral centres, such as celobiose and lactose) can give rise to

differences, both in their chemical reaction and in their behavior in the purification processes. Raymond Lemieux summed it up very well with the following phrase: *"The only generalization that exists in the chemistry of carbohydrates is that there is no generalization.* " Applicants submit herewith two additional journal articles for the Examiner's consideration that each further describe some of the complexities and problems associated with carbohydrate chemistry. The journal articles are Marcaurelle *et al., Current Opinion in Chemical Biology*, 2002, 6:289-296 and Holemann *et al., Current Opinion in Biotechnology*, 2004, 15:615-622. As such, there can be no reasonable expectation of succeeding with the instantly claimed process.

Regarding crystallization

The Examiner says that crystallization is a common procedure for the purification of saccharides. and that one of ordinary skill in the art would recognize that recrystallization is a common process for isolating saccharides. Further, the Examiner says that the choice of solvent is a matter of routine optimization absent evidence of unexpectedly superior results. Applicants submit that although crystalization is in fact a common process for purifying sugars, nevertheless, finding the appropriate solvent is not easy. It depends on the type of molecules and the range of solvents to be tested can be very broad. The more customary solvents in sugars tend to be low molecular weight alcohols, water, ethyl acetate, hexane, and their mixtures (Ponpipom et al. use mainly alcohols). Crumpton *et al.* use acetone. Applicants respectfully submit that in a crystallization, one of ordinary skill in the art needs to try out a large number of solvents and mixtures thereof before arriving at the appropriate solvent. In the instant case the use of acetone allows obtaining the product desired with a >99% degree of purity, which was not possible with the more usual solvents. As such, the references cited provide no more than an invitation to try various solvents, and an invitation to try has been found insufficient by the courts to establish *prima facie* obviousness. Even if, *assuming arguendo*, a *prima facie* case of obviousness had been established, the unexpectedly superior >99% degree of purity is sufficient to rebut the same.

2. *Reyes et al., U.S. Patent 5,994,092 in view of Ponpipom et al., U.S. Patent 4,228,274 and Crumpton et al., Biochem. J. 70(4):729 (1958) further in view of Wong-Madden et al., U.S. Patent 5,770,405 and Dahmen et al., U.S. Patent 4,675,392*

The Examiner rejects claims 1, 5, 6 and 16-19 as unpatentable over Reyes *et al.*, U.S. Patent 5,994,092 in view of Ponpipom *et al.*, U.S. Patent 4,228,274 and Crumpton *et al.*, *Biochem. J.* 70(4):729 (1958) further in view of Wong-Madden *et al.*, U.S. Patent 5,770,405 and Dahmen *et al.*, U.S. Patent 4,675,392. The Examiner appears to admit that Reyes *et al.* do not teach using a water/isopropanol gradient to elute 4GPX. However, Reyes *et al.* allegedly teach water/ethanol and Wong-Madden *et al.* allegedly show that isopropanol is an art recognized equivalent.

The Examiner further admits that none of the references teach the particulars of the solvent gradient in claim 17 or the amount of activated carbon in claim 18. However, the Examiner alleges that these are variables that are optimized by routine experimentation. As for employing water/isopropanol as eluent, Applicants respectfully submit that Wong-Madden et al., U.S. Patent 5,770,405 do not use the solvent mixture in a chromatography on active carbon. In column 33, line 25, Wong-Madden et al. teach using isopropan/ethanol/water, but it is to develop a chromatography on silica gel. As such, there is no teaching or suggestion for combining the references as the Examiner has done. Therefore, the Examiner has failed to set forth a *prima facie* case of obviousness.

Active carbon is customarily used to eliminate hydrophobic impurities, but it is not normally used for separating monosaccharide and disaccharide mixtures, such as is the case in the instant invention. To separate these mixtures the normal course is to employ cmomatography on silica gel, on sepharose or others (See, Wong-Madden et al., Column 11, line 19). The present invention describes purifying a mono- and disaccharide mixture using active carbon, which offers the advantage, compared with usual adsorbents (silica gel or sepharose, for example) of being cheaper.

As regards selection of the eluent, the process of the instant invention uses isopropanol/water mixtures, as opposed to the usual alcohol/water mixtures, such as methanol/water or ethanol/water. The present invention thereby provides the advantage of allowing for less elution volume, a significant advantage for industrial production. Moreover, ethanol and methanol are more toxic.

3. *Reyes et al., U.S. Patent 5,994,092 in view of Ponpipom et al., U.S. Patent 4,228,274 and Crumpton et al., Biochem. J. 70(4):729 (1958) further in view of Wong-Madden et al.,*

U.S. Patent 5,770,405 and Dahmen et al., U.S. Patent 4,675,392 and further in view of Rao et al., Qual. Plant.-Pl. Fds. Hum. Nutr. XXVIII 4:293-303 (1979)

The Examiner rejects claims 1 and 7-15 as unpatentable over Reyes et al., U.S. Patent 5,994,092 in view of Ponpipom et al., U.S. Patent 4,228,274 and Crumpton et al., Biochem. J. 70(4):729 (1958) further in view of Wong-Madden et al., U.S. Patent 5,770,405 and Dahmen et al., U.S. Patent 4,675,392 and further in view of Rao et al., Qual. Plant.-Pl. Fds. Hum. Nutr. XXVIII 4:293-303 (1979). The Examiner admits that Reyes et al. do not teach using celite in the reaction mixture and extraction of the disaccharide with a Soxhlet extractor. However, the Examiner says that Rao et al. teach Soxhlet extractors and celite. The Examiner adds that it would be a matter of routine optimization by one of ordinary skill in the art to select solvents known in the art that dissolved saccharides for Soxhlet extraction absent any evidence to the criticality of solvent selection for the extraction or teaching of unexpected results. Further, the Examiner adds that it would be routine to optimize the amount used. Finally, the Examiner that the deactivation with HCl is routine optimization.

Regarding extraction with Soxhlet, Rao et al. teach this process to extract fats from a specimen with plant origin. There is no teaching or suggestion of using Soxhlet for selectively extracting monosaccharides from a mixture of sugars. As such, there is no teaching or suggestion for combining the references as the Examiner has done. Therefore, the Examiner has failed to set forth a *prima facie* case of obviousness.

The Examiner is right in that HCl for this purpose is routine, however, no claim rests on this feature alone for patentability.

4. *Reyes et al., U.S. Patent 5,994,092 in view of Ponpipom et al., U.S. Patent 4,228,274, Crumpton et al., Biochem. J. 70(4):729 (1958), Dahmen et al., U.S. Patent 4,675,392, Rao et al., Qual. Plant.-Pl. Fds. Hum. Nutr. XXVIII 4:293-303 (1979) and Wong-Madden et al., U.S. Patent 5,770,405 in further view of Gabelsberger et al., FEMS Leters 109(2-3): 131 (1993), Fujimoto et al., Glycoconjugate Journal 15:155 (1998) and Yoshitake et al., Eur. J. Biochem. 101:395 (1979).*

The Examiner rejects claims 25 and 26 as unpatentable over this combination. The Examiner admits that Reyes et al. and the other primary references do not teach adding cosolvents DMF, DMSO and dioxane. However, all of the other three secondary references

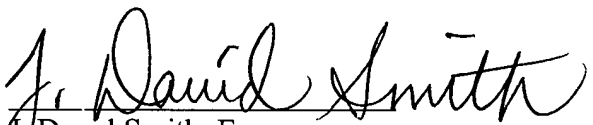
allegedly use these cosolvents in forming a phosphate buffer. According to the Examiner, it would have been obvious to one of ordinary skill in the art to use any of these three solvents in the phosphate buffer of Reyes et al. Further, the amounts recited represent mere optimization parameters according to the Examiner.

Concerning the use of cosolvents, Applicants submit that this can be considered routine and, moreover, when cosolvents are added to the reaction medium the yields are lower. However, no claim rests on this feature alone for patentability.

CONCLUSION

Entry of the foregoing remarks into the record of the above identified application is respectfully requested. It is believed that all of the claims are in condition for allowance. If any issue can be resolved telephonically, the Examiner is requested to call the undersigned at the phone number provided.

Respectfully submitted,



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ELSEVIER

Carbohydrate diversity: synthesis of glycoconjugates and complex carbohydrates

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The fundamental role of glycoconjugates in many biological processes is now well appreciated and has intensified the development of innovative and improved synthetic strategies. All areas of synthetic methodology have seen major advances and many complex, highly branched carbohydrates and glycoproteins have been prepared using solution- and/or solid-phase approaches. The development of an automated oligosaccharide synthesizer provides rapid access to biologically relevant compounds. These chemical approaches help to produce sufficient quantities of defined oligosaccharides for biological studies. Synthetic chemistry also supports an improved understanding of glycobiology and will eventually result in the discovery of new therapeutics.

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Current Opinion in Biotechnology 2004, 15:615–622

This review comes from a themed issue on
Chemical biotechnology
Edited by Ronald Frank

Available online 22nd October 2004

0958-1669/$ – see front matter

DOI 10.1016/j.copbio.2004.10.001

Abbreviations

GlcNAc	*N*-acetylglucosamine
GPI	glycosylphosphatidylinositol
HIV	human immunodeficiency virus
PSA	prostate-specific antigen

Introduction

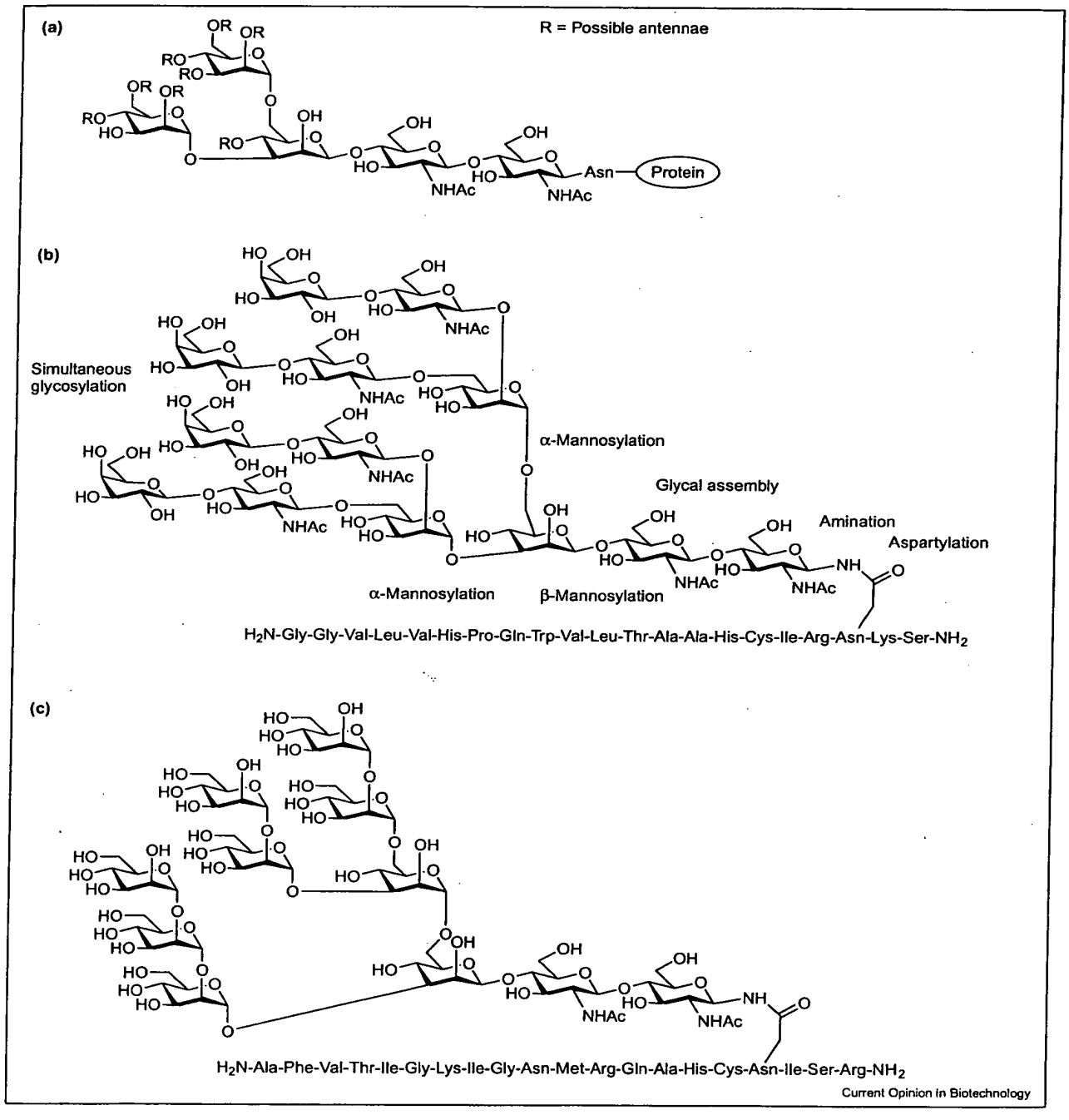
In addition to oligopeptides and oligonucleotides, oligosaccharides (glycans) constitute the third major class of naturally occurring biopolymers that play a fundamental role in many important biological processes. Glycans are commonly found in nature as glycoconjugates (glycoproteins or glycolipids) that show high structural diversity, greatly exceeding the diversity of proteins and nucleic acids.

In contrast to linear oligopeptides and oligonucleotides, oligosaccharides are often complex branched molecules and the glycan core is commonly attached to proteins and lipids. In nature, three major classes of glycans exist: *N*-linked glycans, *O*-linked glycans and glycosylphosphatidylinositol (GPI) anchors. Intensive research into the biological role of carbohydrates has led to an increased need for sufficient quantities of natural and modified glycoproteins; however, the isolation of carbohydrates from natural sources is extremely difficult owing to their structural complexity. Access to pure carbohydrates for biological, biochemical, biophysical and medicinal studies therefore relies on chemical and enzymatic synthesis [1,2]. Remarkable progress has been made in this area; however, further innovations are required to handle the structural complexity of oligosaccharides. Their preparation is technically difficult, extremely time-consuming and performed by a few specialized laboratories. The introduction of solid-phase synthesis strategies has significantly improved carbohydrate assembly, as an excess of reagent can be used to ensure high yields and to reduce the number of purification steps. The development of an automated oligosaccharide synthesizer [3•,4•,5••] has led to rapid access to complex carbohydrates of biological relevance. This review highlights recent advances in the synthesis of complex oligosaccharides and glycoproteins, primarily focusing on strategies published in the past two years.

N-Linked glycoproteins

N-Linked glycoproteins (*N*-glycans) are the most abundant in nature and are commonly divided into four groups: high-mannose, complex, hybrid and poly-*N*-acetyllactosamine glycans. Although the structural details are well established, little is known about their structure–activity relationship. In *N*-glycans, the oligosaccharide sidechain is attached to the protein via an asparagine amino acid. All *N*-glycans share the common pentasaccharide core structure (mannose)$_3$(*N*-acetylglucosamine)$_2$ ($Man_3GlcNAc_2$) shown in Figure 1a. Structural diversity is generated by variation in the substitution pattern of the pentasaccharide core, in the degree of branching and in the terminal sugars. The pentasaccharide core can be extended by up to five antennae. The preparation of the basic structure contains several synthetic challenges, including branching and the inclusion of a β-mannoside. Recently, two efficient partial syntheses of the core structure have been accomplished [6,7], selectively establishing the β-mannosidic linkage. The orthogonally protected β-mannosylated chitobiose trisaccharide with a terminal azido group serves as a key building block in the preparation of complex *N*-glycans. The entire pentasaccharide has been synthesized recently by Danishefsky and colleagues [8]

Figure 1



N-Linked glycoproteins. **(a)** Structure of the core pentasaccharide common to all *N*-glycans. The core structure can be extended by up to five antennae (R). **(b)** Structure of a prostate-specific antigen (PSA) glycopeptide. The crucial retrosynthetic steps of Danishefsky's [21•] strategy are shown. **(c)** Structure of the gp120 glycopeptide fragment, which is a possible target for an anti-HIV-vaccine. Protein sequences are shown using the three-letter amino acid code.

using Crich's β-mannosylation methodology [6] followed by a simultaneous di-α-mannosylation with a thiomannoside donor.

As an alternative to these solution-phase preparations, the synthesis of the core pentasaccharide selectively functionalized with one *N*-acetylglucosamine residue has been performed recently using a solid-phase approach [9]. The first automated solid-phase oligosaccharide synthesizer [5••] has been used to efficiently prepare the core pentasaccharide [10] by using an octenediol functionalized Merrifield's resin and three different building blocks: two monosaccharides and one disaccharide already containing the β-mannosidic linkage. Branching was achieved by simultaneous dimannosylation of the trisaccharide core.

Innovative synthetic methods have also provided access to more complex and highly branched *N*-glycans. Weiss and Unverzagt [11] have developed a general strategy for the preparation of multiantennary *N*-glycans. Crucial challenges in the synthesis of these sterically crowded bi- to tetra-antennary compounds is the sequence of introducing the building blocks and the steric demand of the building blocks. Complex biantennary *N*-glycans are also accessible via chemoenzymatic total synthesis. Elongation of synthetic oligosaccharides has been performed using glycosyltransferases to give full-length *N*-glycans [12,13].

Synthetic oligosaccharides are useful in gaining a more detailed understanding of glycoprotein quality control. In particular, maintenance of the integrity of protein folding has recently received significant attention. Ito and colleagues [14,15] accomplished a convergent and stereoselective route to the nonasaccharide $Man_8GlcNAc_2$ and the monoglucosylated dodecasaccharide α-$Glc_1Man_9GlcNAc_2$, a putative ligand of the molecular chaperones calnexin and calreticurin. These synthetic oligosaccharides might serve as molecular probes to detect glycoprotein–mannosidase-like protein recognition.

Glycoproteins are also important in the context of diagnostics, therapeutics and vaccines. The integration of oligosaccharides into glycoproteins is realized by converting them into anomeric glycosylamines, which is either performed by treatment with ammonium hydrogencarbonate or by reduction of anomeric glycosyl azides, and subsequent attachment to the peptide chain [16–18]. Guo and colleagues [19] attached a fucoslyated trisaccharide to the peptide of the CD52 antigen by using a solution-phase synthesis with solid-phase workup or a combined solution- and solid-phase approach. More complex oligosaccharides containing two thiol residues were linked to the same peptide by 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase peptide synthesis [20]. Because of their short peptide chain containing only 12 amino acids, their simple glycosylation pattern and their interesting bioactivity, these glycopeptides serve as useful models to study structure–activity relationships. The development of a universal strategy [21•] for the preparation of complex multibranched *N*-acetyllactosamine-type glycans from common precursors has led to the first chemical synthesis of normal and transformed prostate-specific antigen (PSA) glycopeptides (Figure 1b). PSA has been identified as a highly specific cancer marker that might enable the early diagnosis of prostate tumours.

N-Linked carbohydrates also play an important role in human immunodeficiency virus (HIV) retroviral pathogenesis. The HIV-1 surface envelope glycoprotein gp120 is highly glycosylated containing up to 24 *N*-linked high-mannose carbohydrates and shows biological functions in helper T-lymphocyte infections [22]. Seeberger and colleagues [23] developed a linear solution-phase synthesis of a triantennary high-mannose nonasaccharide from gp120 using just three monosaccharide building blocks. Employing a reactivity-based one-pot self-condensation approach, Wong and coworkers [24] prepared several high-mannose oligosaccharides, which efficiently inhibit the binding of the antibody 2G12 to gp120. More recently, Danishefsky and colleagues [25••,26••] described the first chemical synthesis of HIV gp120 fragments (Figure 1c), which serve as targets for an anti-HIV vaccine.

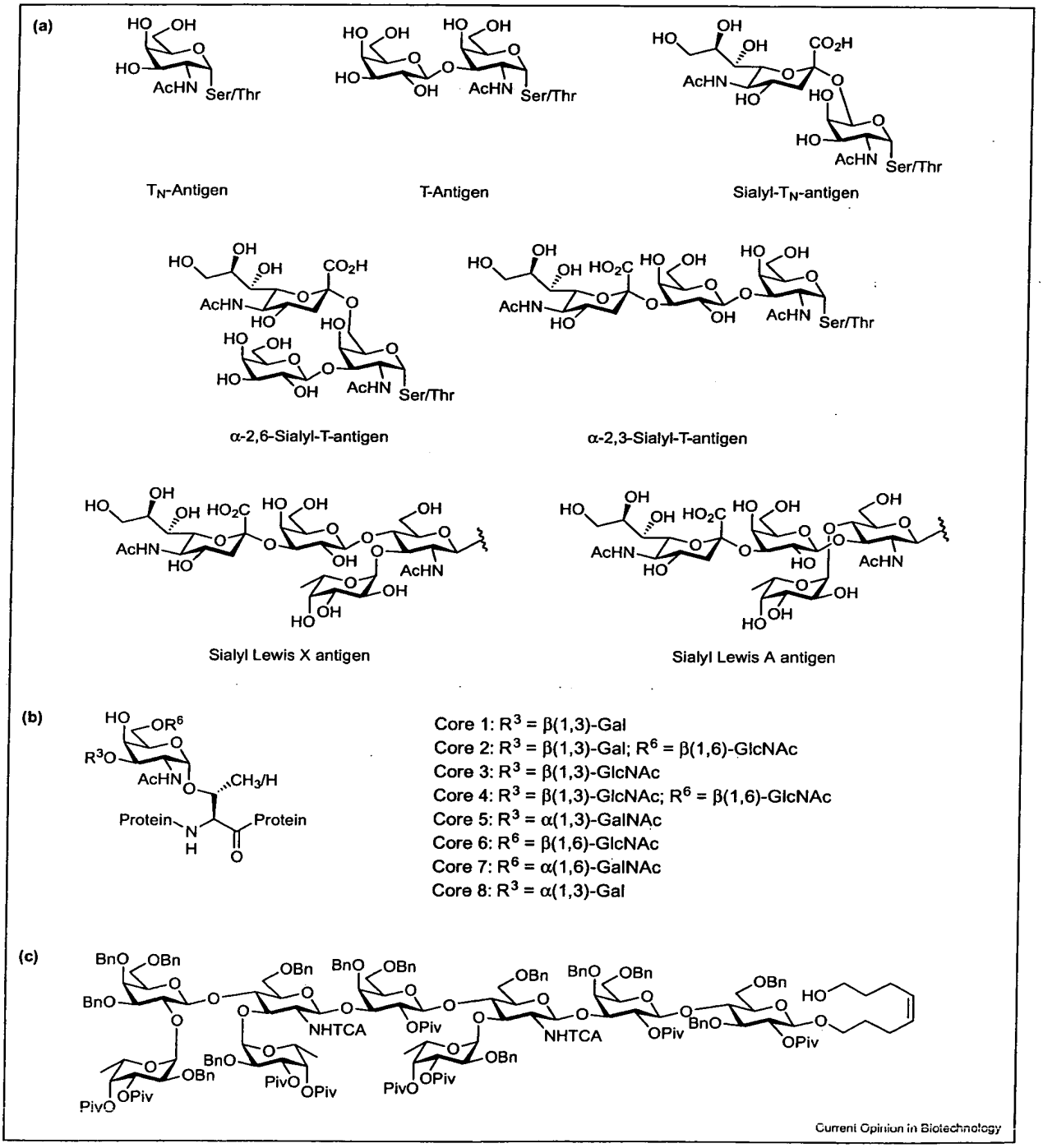
Many important glycoproteins are multiply glycosylated at fixed sites. Danishefsky's laboratory [27] recently disclosed a convergent method for the preparation of bifunctional glycopeptides: two glycopeptides are synthesized separately from their glycan and peptide precursors using standard procedures and subsequently coupled to yield the bifunctional compounds.

O-Linked glycoproteins

A second major group of biologically important glycoproteins are *O*-linked glycoproteins (*O*-glycans). The carbohydrate residue in *O*-glycans is covalently attached to the peptide backbone via the hydroxyl group of serine, threonine, tyrosine, hydroxyproline, hydroxylysine or another hydroxylated amino acid. In contrast to *N*-glycans, these glycoproteins show a higher degree of structural diversity and do not share a common core structure. Additional variety arises from further carbohydrate elongations of these backbones.

Tumour-associated antigens (Figure 2a) like the T_N-, T-, sialyl-T_N and sialyl-T antigens as well as the sialyl Lewis X and sialyl Lewis A antigens were first found in mucins. Mucins are a class of highly *O*-glycosylated proteins present on the surface of various types of epithelial cells. In normal tissue, the peptide backbone carries several complex oligosaccharides derived from the glycan core structures shown in Figure 2b, which are characterized by

Figure 2



O-Linked glycoproteins. **(a)** Structures of tumour-associated carbohydrate antigens that were first discovered in mucins. **(b)** Core structures of mucin-type *O*-linked glycans, a class of highly *O*-glycosylated proteins. **(c)** Structure of the Le^y-Le^x tumour marker. Ac, acetyl; Bn, benzyl; Piv, pivaloyl; TCA, trichloroacetyl.

an *N*-acetylgalactosamine unit α-*O*-linked to serine or threonine. An increased expression of mucins is usually prevalent in tumour cells, where the carbohydrate chains are modified due to incomplete glycosylation and premature sialylation. As tumour-associated glycans with peptide sequences of mucins constitute a promising target for the development of synthetic antitumour vaccines, the chemical synthesis of such glycoconjugates has received considerable attention and several reviews devoted to this field of research have been published [28–30,31•].

A solid-phase approach [32,33] has been used for the stereoselective construction of several different mucin-type *O*-glycans. Stepwise elongation of the carbohydrate led to the required highly glycosylated amino acid building blocks, which were then incorporated into a solid-phase glycopeptide synthesis. Other branched *O*-glycans have recently been prepared by an efficient one-pot glycosylation approach using either glycosyl fluoride [34] or thioglycoside [35] building blocks. As sialylated derivatives of tumour-associated antigens are also present on the surface of cancer cells, the preparation of *O*-linked sialyl oligosaccharides is important. Paulson and colleagues [36] demonstrated that recombinant sialyltransferases are ideal catalysts for the simple and efficient preparation of *O*-linked sialyl oligosaccharides by elongation of a synthetic glycosyl amino acid.

The application of non-natural amino acids in carbohydrate vaccines has also attracted considerable attention [37], as these unnatural linkages might give an increased immune response. Danishefsky's group [38] investigated the synthesis of different glycosyl hydroxynorleucines, each containing a tumour-associated carbohydrate antigen. While the glycosylation of trichloroacetimidate donors with the amino acid predominately afforded the corresponding α-*O*-linked product, the reaction with a glycal epoxide donor provided the β-*O*-linked product. The glycal methodology was also successfully applied to the synthesis of Lewis Y- and Globo-H-containing amino acids.

More recently, an automated synthesizer has been used to accelerate the synthesis of the Lewis^{y}-Lewis^{x} tumour marker (Figure 2c) and the Lewis X and Lewis Y blood group antigens [39••]. Only five monomers were necessary for the efficient construction of the three target structures.

GPI anchors

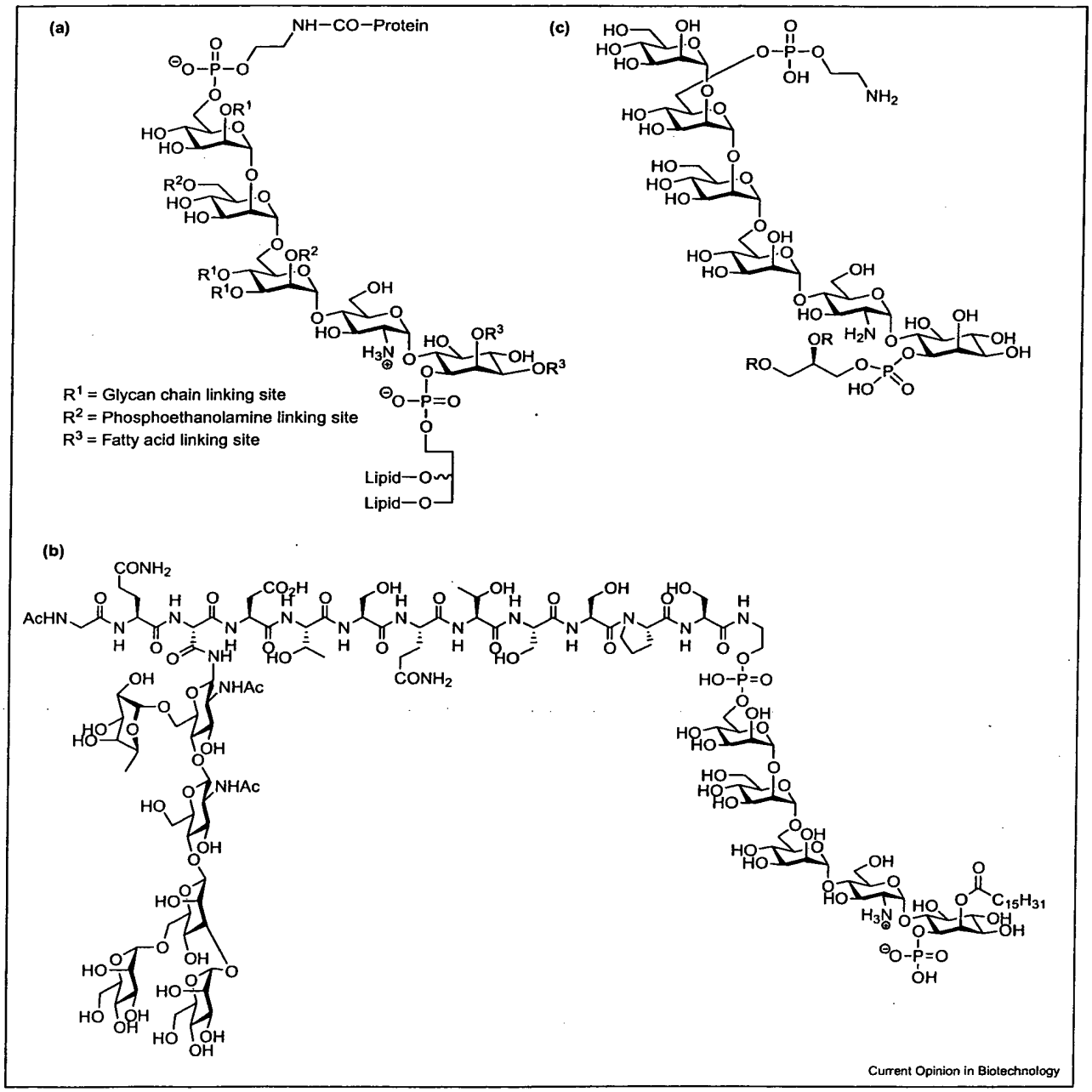
GPI-anchored proteins are involved in many biological and physiological processes and have attracted considerable attention since the first structure determination of a GPI in 1988 [40]. These naturally occurring glycolipids serve to attach proteins or glycoproteins onto eukaryotic cell membranes. All reported GPI structures share the basic core structure shown in Figure 3a with a linear tetrasaccharide attached to the 6-*O*-position of inositol. Besides this conserved general structure, considerable diversity exists within the GPI anchor family based on the variation of the substitution pattern on this pseudo-pentasaccharide backbone. In most cases, the core is further modified by species-specific carbohydrates, additional phosphoethanolamine units and variations in the lipid moiety. Proteins or glycoproteins are linked to the non-reducing end by their C termini or a phosphoethanolamine group. Owing to the structural complexity of the GPI anchors that requires a detailed knowledge of lipid, phosphate and oligosaccharide chemistry, many chemists have focused on the synthesis of these motifs [41•].

A linear solution-phase approach allows for the construction of complex GPI anchors and for the preparation of an orthogonally protected derivative of the phosphorylated pseudo-pentasaccharide core [42]. Another variable concept for the preparation of branched GPIs was developed by Pekari and Schmidt [43]. The efficiency of this approach was demonstrated by the synthesis of the GPI anchors of rat brain Thy-1 and scrapie prion protein in their water-soluble and lipidated forms. This approach also allows further attachment of peptide residues or biological markers to the GPI anchor. Reichardt and Martin-Lomas [44] reported a soluble support-based approach for the synthesis of the GPI backbone. This method, using a polyethylene glycol-grafted polystyrene resin functionalized with a Wang-chloride linker, can be applied to the preparation of a small library of GPI precursors.

CD52 antigens, simple GPI-anchored glycopeptides, are present on eukaryotic cells and play an important role in the human immune system. Initial studies aimed at the synthesis of sperm CD52, including the preparation of an acylated inositol [45] and the linkage to the peptide [46], were performed by Guo and colleagues. More recently, they reported [47••] the first synthesis of a skeleton structure of sperm CD52. In their strategy the glycopeptide and the GPI anchor were prepared separately and subsequently linked by an amide bond to give the glycopeptide–GPI conjugate (Figure 3b).

Synthetic GPIs are promising vaccine candidates against malaria, as shown in a mouse model [48]. Annually, malaria infects 5–10% of the world's population and kills about 3 million people each year. The malaria parasite *Plasmodium falciparum* expresses a large amount of GPI anchored to a protein, and the GPI structure (Figure 3c) has been identified as the malaria toxin. A solution-phase synthesis of two malaria vaccine candidates with a pseudo-hexasaccharide backbone has recently been reported by Seeberger and collagues [49]. This strategy allows for scale-up to procure compounds for preclinical

Figure 3



GPI anchors. **(a)** Basic core structure of all GPI anchors. **(b)** Skeleton structure of sperm CD52. **(c)** Structure of the malaria GPI vaccine candidate.

and clinical trials. The authors also demonstrated that the synthesis of this target can be automated effectively [50••]. The fully protected oligosaccharide was obtained in only 9 h starting from four monosaccharides and one disaccharide building block. Fraser-Reid and coworkers [51,52••] developed a method for the solution-phase

synthesis of a fully lipidated and phosphorylated malarial GPI pseudo-pentasaccharide using orthoesters and methyl α-D-glucopyranoside as the key building blocks.

Conclusions

Innovative synthetic methods are an important tool to create diverse carbohydrates. Recent advances in the preparation of complex oligosaccharides as well as entire glycoproteins containing *N*-glycans, *O*-glycans and GPI anchors have been highlighted in this review. Highly branched carbohydrates and biologically relevant oligosaccharides are now accessible via these methods, providing sufficient quantities for biological studies. The availability of defined synthetic glycoproteins and glycolipids will significantly support biological investigations. The development of new strategies for the preparation of carbohydrates is fundamental for the understanding of carbohydrate–protein interactions, biosynthetic pathways and structure–activity relationships and will allow for the discovery of new targets for therapeutics, diagnostics and vaccines. The introduction of an automated oligosaccharide synthesizer has greatly accelerated access to many highly branched carbohydrates, and a series of biologically relevant oligosaccharides has been efficiently prepared on this machine. Further improvement and extension of this technology could allow for the automated synthesis of complex glycoproteins, proteoglycans and glycolipids using only one instrument and will eventually enable even non-specialists to create biologically important compounds for biochemical, biophysical and medicinal applications.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

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Combinatorial carbohydrate chemistry

Lisa A Marcaurelle and Peter H Seeberger*

The application of combinatorial chemistry to the synthesis of carbohydrate-based compound collections has received increased attention in recent years. New strategies for the solution-phase synthesis of oligosaccharide libraries have been reported, and the use of monosaccharides as scaffolds in the generation of combinatorial libraries has been described. Novel approaches to the assembly of carbohydrate-based antibiotics, such as aminoglycoside analogs and vancomycin derivatives, have also been disclosed.

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Current Opinion in Chemical Biology 2002, 6:289–296

1367-5931/02/$ – see front matter

Published online 20 March 2002

Abbrevation
RGD Arg–Gly–Asp

Introduction

Combinatorial chemistry has become an important tool in modern drug development. Although carbohydrate-based compounds hold great potential as therapeutic agents, the application of combinatorial chemistry to this class of biomolecules has only recently elicited attention. The challenges associated with carbohydrate synthesis, including laborious protecting group manipulations and the need for regioselective and stereoselective glycosylation reactions, are primarily responsible for the lack of more intense efforts. The high degree of functionalization and diverse stereochemistry of carbohydrates, the very properties that render them attractive members of compound libraries, are responsible for the complications encountered by the experimentalist. In addressing and overcoming these challenges, the synthesis of a number of carbohydrate-based libraries has been achieved. This review highlights recent progress in the combinatorial synthesis of carbohydrates, including the development of new carbohydrate-based antibiotics and the use of carbohydrates as scaffolds for the synthesis of stereodiverse libraries. Recent advancements in solid-phase oligosaccharide synthesis and its application to carbohydrate libraries is also discussed.

Several excellent articles reviewing combinatorial carbohydrate synthesis have appeared prior to 2000 [1•,2,3]. This article focuses primarily on strategies reported in the past two years. The synthesis of glycopeptide libraries and related glycoconjugates has been reviewed recently and thus will not be covered [4••,5].

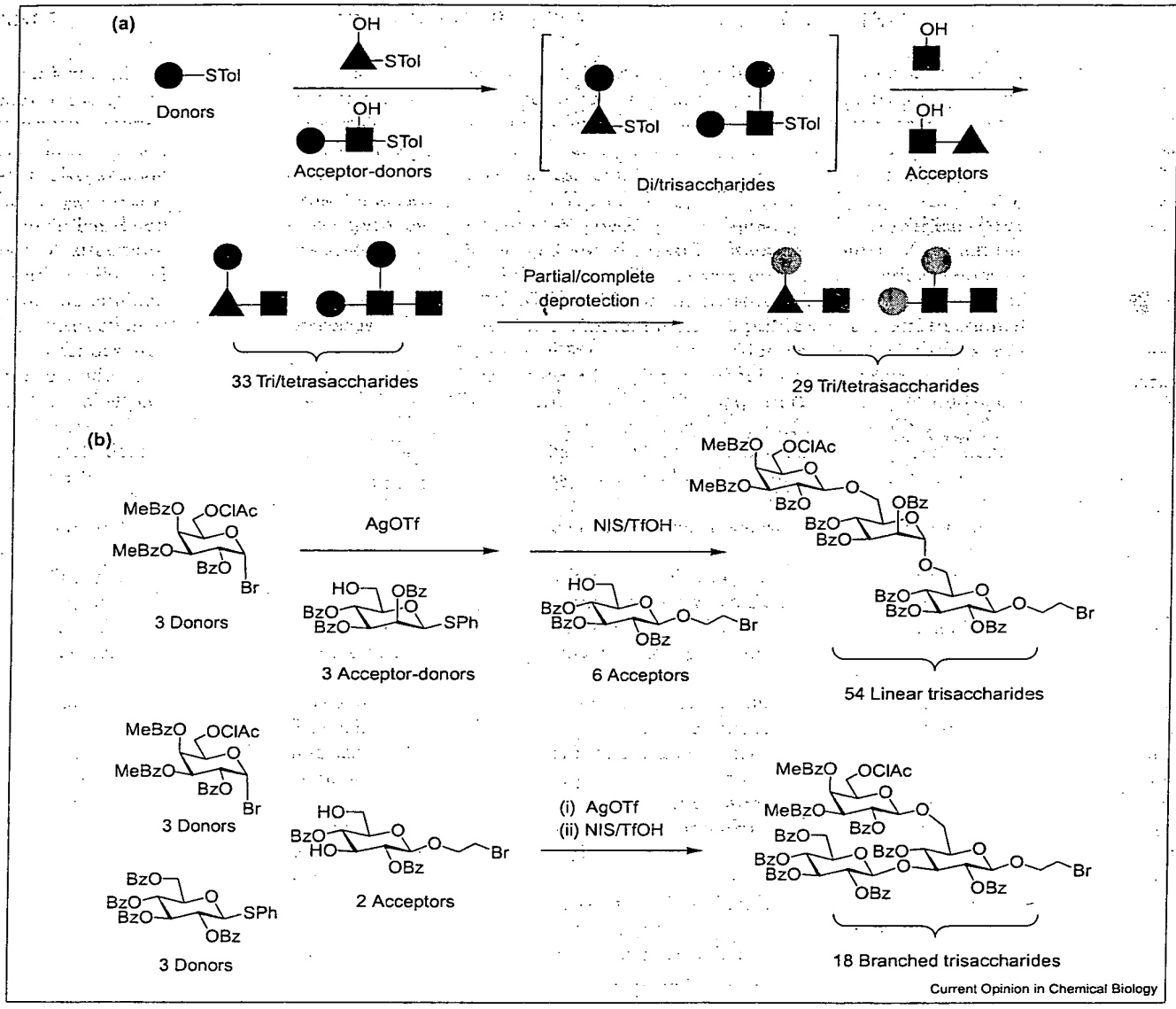
Combinatorial oligosaccharide libraries

In the past five years, the combinatorial synthesis of oligosaccharide libraries has been carried out both in solution and on solid support [1•,2,3]. During the period covered by this review, only two approaches to the combinatorial synthesis of oligosaccharides have been reported [6••,7•]. Both reports describe similar solution-phase approaches, employing sequential one-pot glycosylation strategies. Ye and Wong [6••] made use of their programmable one-pot glycosylation technology [8], which has been employed for the synthesis of a number of structures, including the tumor-associated hexasaccharide Globo-H [9•]. With the aid of anomeric reactivity values determined with the computer program OptiMer™, the construction of a small library of trisaccharides and tetrasaccharides was accomplished using a panel of monosaccharide and disaccharide donors. The sequential reaction of thioglycosides of varying reactivity produced a library of 33 oligosaccharides, which were partially or completely deprotected to create 29 additional compounds (Figure 1a).

In the second approach, Takahashi *et al.* [7•] reported the rapid assembly of a library of linear and branched trisaccharides by using a combination of donors, including glycosyl bromides, thioglycosides and 2-bromoethyl glycosides (Figure 1b). Selective activation of the bromide and thioglycoside donors with AgOTf and NIS/TfOH, respectively, enabled the generation of a library of 72 trisaccharides by sequential one-pot reactions on a manual synthesizer. It should be noted that each member of the library contains two sites for further elaboration. The chloroacetate group can be selectively removed for attachment of the trisaccharide to solid-support, while the bromoethyl glycoside can be modified by alkylation for the introduction of diversity at the anomeric position.

The synthesis of oligosaccharide libraries in solution has been quite fruitful. Still, the use of solid-phase methods for the construction of glycosidic linkages is attractive, because an excess of reagents may be used to ensure high yields and the number of purification steps is reduced. The solid-phase synthesis of oligosaccharide libraries was first reported by Kahne and co-workers [10] and later by Zhu and Boons [11]. Although no new methods for the solid-phase synthesis of oligosaccharide libraries have been reported during the past two years, a number of strategies for the solid-phase synthesis of oligosaccharides in general have been reported [12–14,15••,16•], including the automation of oligosaccharide assembly. The first automated solid-phase oligosaccharide synthesizer [15••] was used to prepare structures as large as branched dodecamers in less than one day. The synthesis was achieved using a re-engineered peptide synthesizer containing a coolable reaction vessel, utilizing glycosyl phosphates and glycosyl trichloroacetimidate building blocks (Figure 2). Each cycle involved the coupling of a building block to a growing resin-bound oligosaccharide and the removal of a protecting group to expose a single hydroxyl

Figure 1



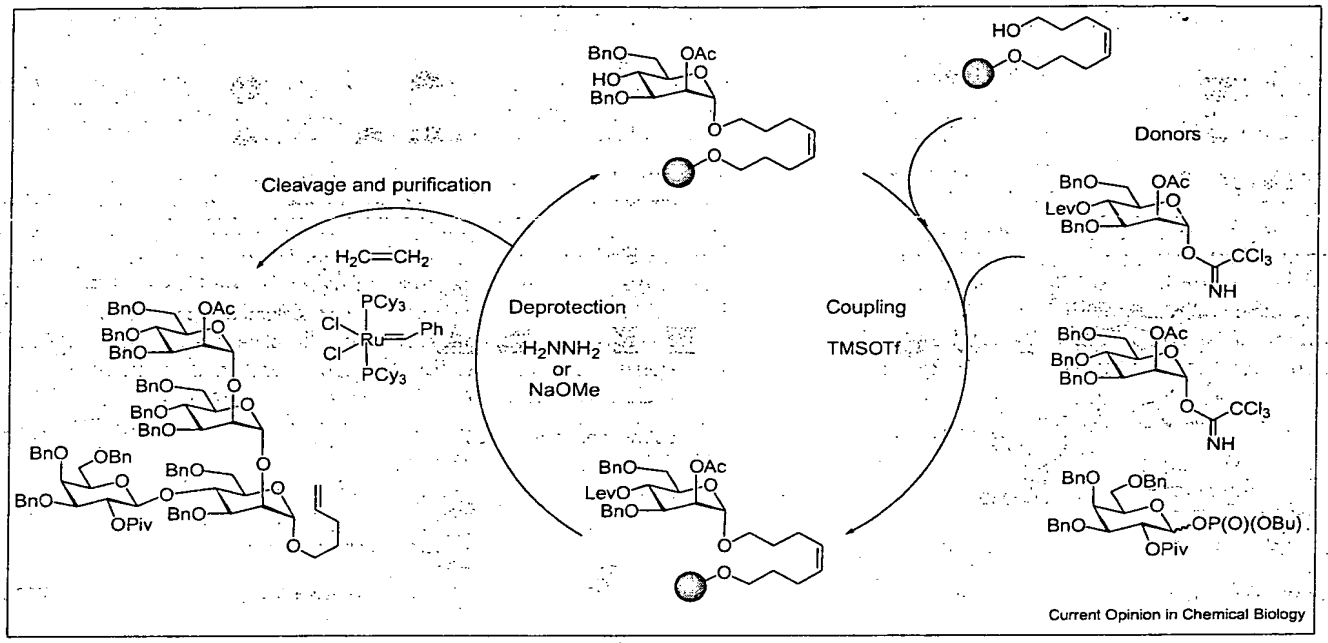
Novel approaches to oligosaccaharide libraries. **(a)** Wong's approach to the one-pot assembly of a library of linear and branched trisaccharides and tetrasaccharides. The sequential reaction of thioglycoside donors of varying reactivity produced a library of 33 oligosaccharides, which were partially or completely deprotected to afford 29 more compounds. **(b)** Takahashi's one-pot sequential assembly of a library of trisaccharides. Selective activation of glycosyl bromide and thioglycoside donors with AgOTf and NIS/TfOH, respectively, yielded a library of 72 linear and branched trisaccharides. Bz, benzoyl group; NIS, *N*-iodosuccinimide; TfOH, trifluoromethanesulfonic acid.

group for attachment of the next carbohydrate. A metathesis-cleavable octenediol linker enabled release of the oligosaccharide from the support using Grubb's catalyst. This method has recently been applied to the synthesis of a branched tetrasaccharide (Figure 2) corresponding to a portion of the cell-surface lipophosphoglycan of *Leishmania* parasites [16•]. Branching of the tetrasaccharide was achieved through the selective removal of different ester protecting groups. The automation of oligosaccharide synthesis is expected to greatly facilitate preparation of oligosaccharide libraries by parallel synthesis.

Carbohydrate scaffolds for combinatorial synthesis

Monosaccharides are particularly attractive scaffolds for the synthesis of combinatorial libraries. They are readily available, conformationally rigid, chiral and highly functionalized molecules, containing up to five hydroxyl

Figure 2



Solid-phase synthesis of a branched tetrasaccharide [16•] using an automated oligosaccharide synthesizer [15••]. Bn, benzyl group. Cy, cyclohexyl group; Lev, levulinoyl group; Piv, pivaloyl group.

groups for the introduction of a diverse range of side chains. A variety of synthetic routes to these scaffolds have been reported [17,18] since carbohydrates were first described as 'privileged platforms' [19,20].

Recently, a focused combinatorial library of 126 mimetics of the Arg–Gly–Asp (RGD) peptidic sequence based on a sugar scaffold was rationally designed aided by molecular modeling [21•]. Although carbohydrate scaffolds had previously served as peptidomimetics, this was the first report of a combinatorial library of this class of compounds. D-Xylose was selected as a scaffold for the introduction of acidic and basic functional groups at various positions in order to achieve a high degree of stereodiversity. The α- and β-allyl glycosides of D-xylose (Figure 3a) were modified by benzylation to yield a total of 14 compounds, composed of a mixture of mono-, di- and trihydroxy derivatives. Alkylation with *t*-butylbromoacetate fashioned the corresponding ester derivatives. Using a 'mix-and-split strategy', the 14 compounds were elaborated into a library of 126 members that were functionalized with various amines. Using this strategy, an RGD mimic was identified that displayed activity equal to a known peptide-based inhibitor (RGDS) of integrin-mediated adhesion. The active compound contained an α-linked *N*-propyl substituent and a carboxylic acid at position 4. This method is now being applied to the synthesis of libraries of other biologically relevant peptidomimetics.

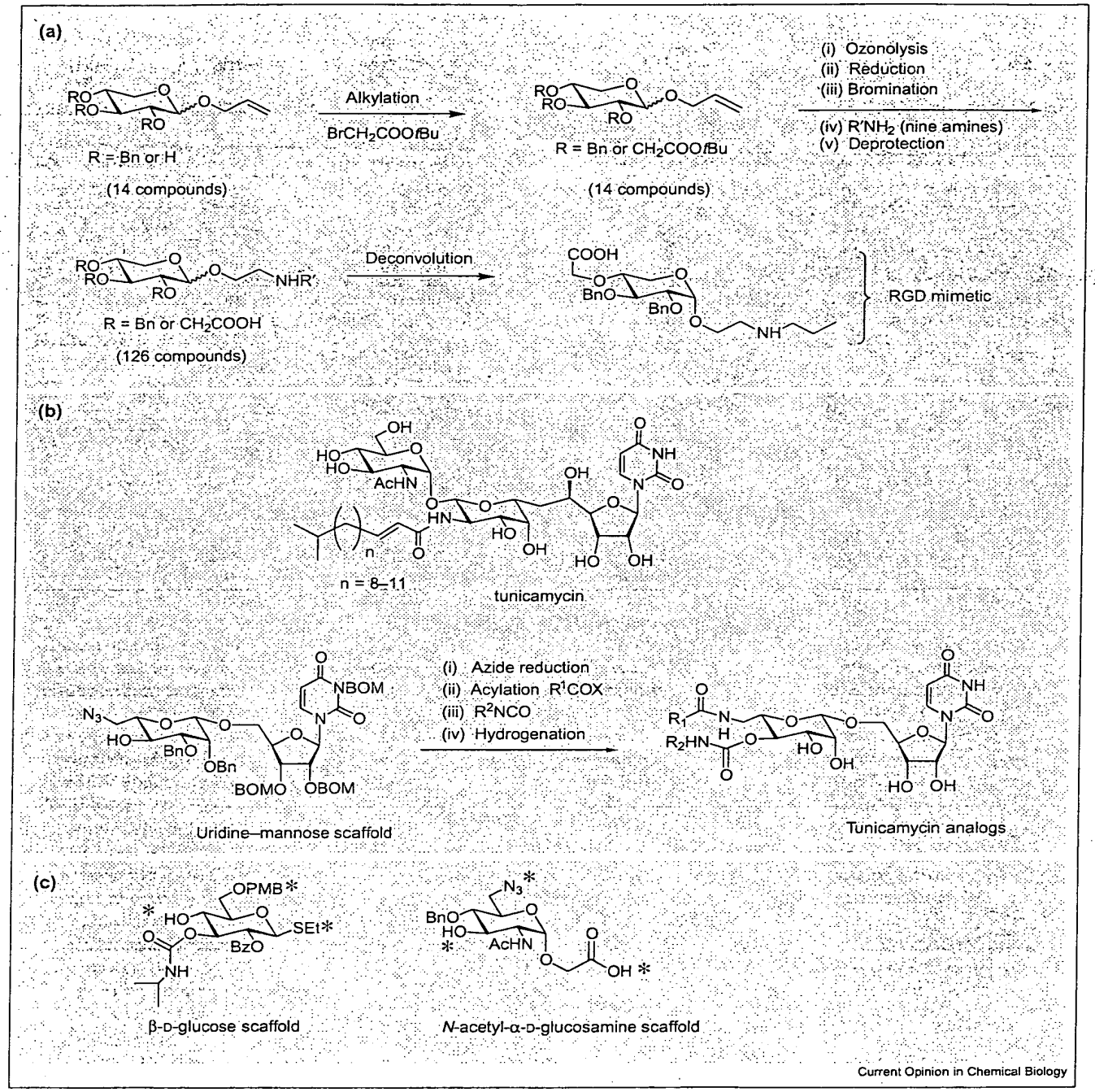
A second recently reported carbohydrate scaffold is based on tunicamycin (Figure 3b; [22]). Tunicamycins have been shown to inhibit a wide variety of lipid carrier-dependent protein glycosylations and are potential antibiotics, as they inhibit bacterial cell wall biosynthesis. Tunicamycins have not been used as therapeutics because they are toxic to mammalian cells, inhibiting all *N*-linked glycosylation. Analogs of tunicamycin may exhibit specific inhibitory effects towards eukaryotic and prokaryotic cells, potentially allowing for the targeting of pathogenic cells over mammalian cells. The tunicamycin scaffold incorporates two sites that can be derivatized orthogonally, an azide and a hydroxyl group (Figure 3b). Following azide reduction, modification of the disaccharide scaffold by acylation and amidation may generate a library of tunicamycin analogs.

Two additional scaffolds have been reported during the past two years (Figure 3c). The β-D-glucose [23] and *N*-acetyl-α-D-glucosamine [24] derived structures were synthesized for the purpose of generating carbohydrate-based libraries for broad screening and can be decorated at the positions indicated by asterisks in Figure 3c. The glucose scaffold is amenable to solid-support synthesis, resulting in an immobilized thioglycoside donor. Further diversity could be generated at the anomeric position by glycosylation.

Libraries of carbohydrate-based antibiotics

A number of antibiotics contain a glycan portion [25••]. Examples of carbohydrate-based antibiotics include

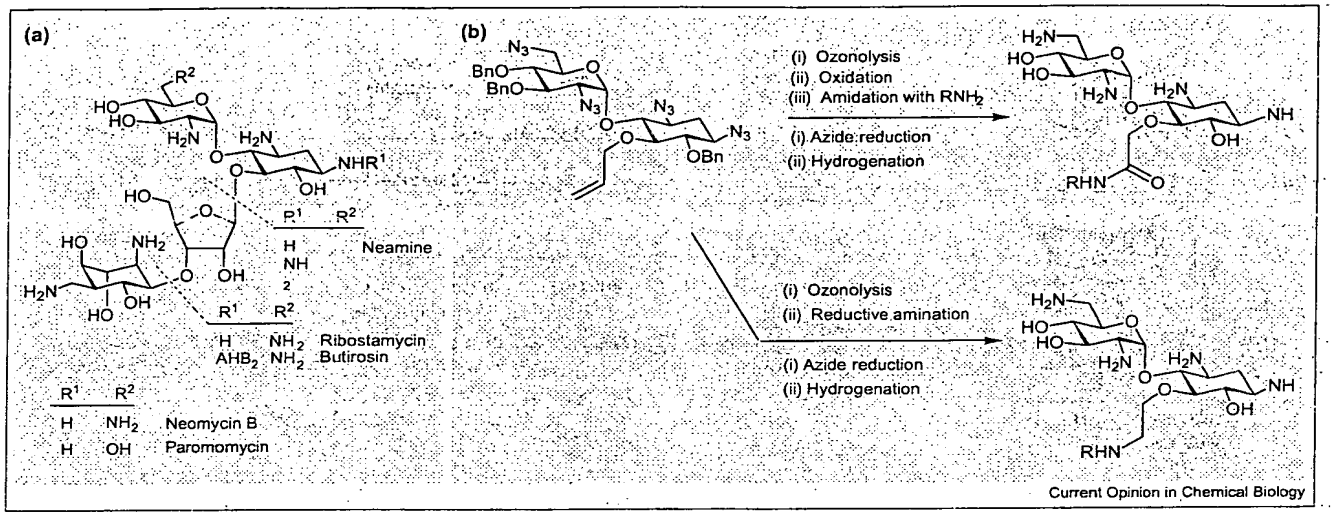
Figure 3



Use of carbohydrates as scaffolds for combinatorial synthesis. **(a)** Synthesis of a combinatorial library of peptidomimetics of the RGD sequence using D-xylose as a sugar scaffold [21•]. An RGD-mimic that was identified from the library is shown. **(b)** Structure of Sofia's carbohydrate scaffold based on tunicamycin containing two sites for functionalization. [22]. **(c)** Structure of carbohydrate scaffolds derived from β-D-glucose [23] and *N*-acetyl-α-D-glucosamine [24]. Sites for functionalization are indicated (*). BOM, benzyloxy methyl; PMB, *para*-methoxybenzyl group.

aminoglycosides, such as neomycin, kanamycin and streptomycin, and the glycopeptides vancomycin and teicoplanin. Because of the recent emergence of a number of drug-resistant bacterial strains, much effort has been focused on the generation of new structures with improved antibiotic activity. Wong and co-workers [26–28] have

Figure 4



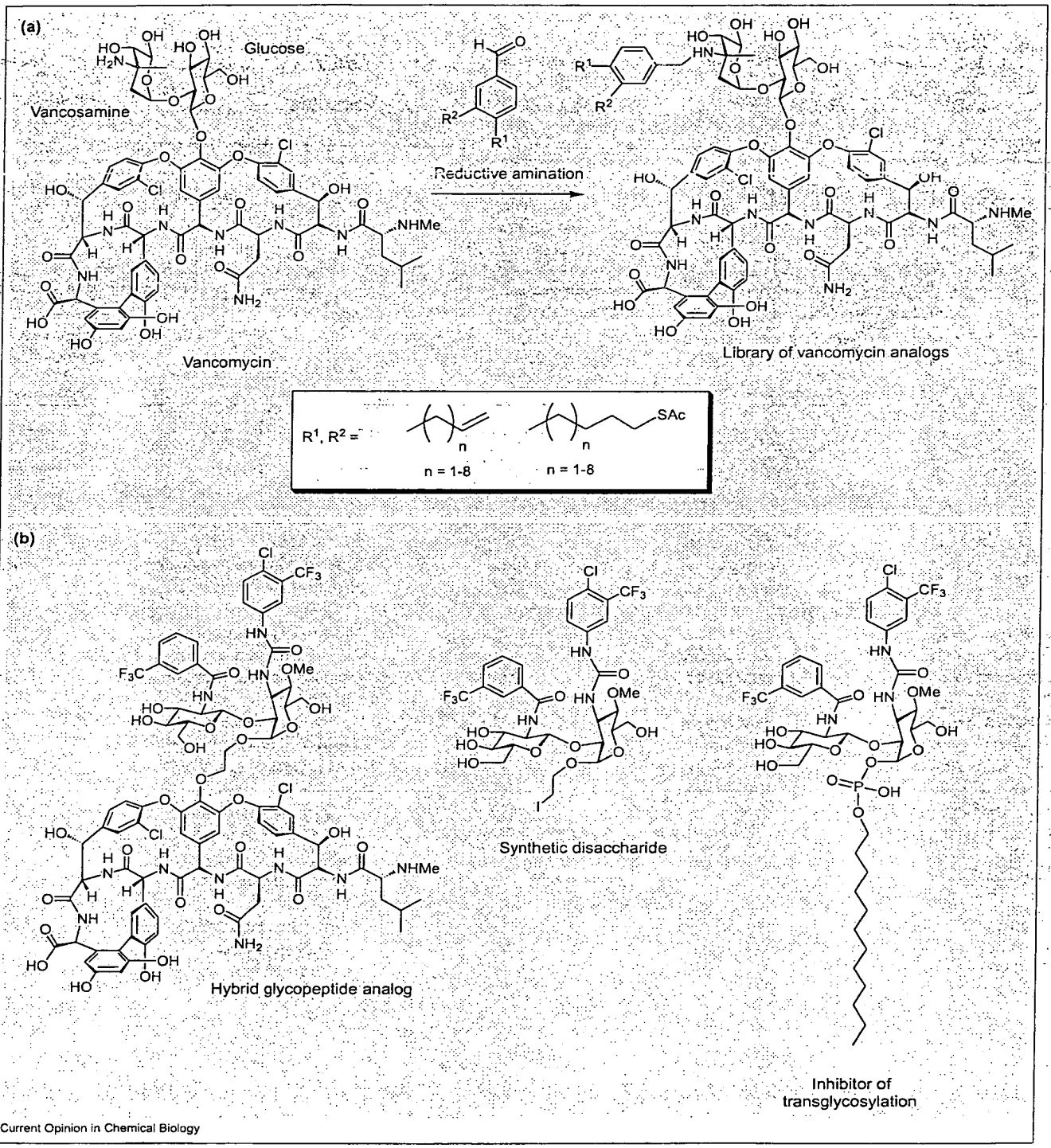
Synthesis of aminoglycoside libraries based on neamine assembled by reductive amination and amidation [29].

reported a number of library-based approaches for the discovery of new aminoglycoside antibiotics. Because of the size and complexity of aminoglycosides such as neomycin (Figure 4), a primary focus for the design of new antibiotics is the identification of simpler structures that retain the activity of the parent compound. Over the course of their studies, Wong *et al.* identified the naturally occurring pseudodisaccharide neamine as a core structure for the generation of new libraries of aminoglycoside mimetics [28]. A recent report described the synthesis of a library of neamine-based compounds and their RNA-binding properties [29]. The neamine library was constructed by reaction of the corresponding azide precursor with a variety of amines after conversion of the 5-*O*-allyl group to a reactive chemical handle (Figure 4). Amidation or reductive amination of the intermediate acid or aldehyde, followed by azide reduction and hydrogenation, yielded a library of compounds modified at the C-5 position of neamine.

The glycopeptide vancomycin (Figure 5a) has been used clinically for the past 40 years to treat infection by Gram-positive bacteria. The emergence of resistance to vancomycin in enterococcal strains has aroused considerable concern [30] and spurred vigorous efforts to develop novel antibiotics to combat these strains. In a series of recent reports, Nicolaou and co-workers [31,32,33••] described the construction of several libraries of vancomycin analogs, modified within the carbohydrate portion of the glycopeptide. Initial efforts were directed towards the replacement of the naturally occurring disaccharide with a panel of synthetic monosaccharides [31]. The glycosylation was performed on solid-phase using trichloracetimidate donors, with the aglycone attached to the resin by a new selenium-based safety-catch linker [32]. The monosaccharide analogs proved to be less active than the parent vancomycin against all bacterial strains. Having established the importance of the vancosamine moiety for antibacterial activity Nicolaou and co-workers turned their attention to the modification of the existing glycan by reductive amination. Reaction of vancomycin with a variety of substituted benzaldehydes (containing terminal alkenes or thioacetates) yielded a library of vancomycin analogs (Figure 5a). Biological evaluation of this library revealed several highly potent compounds effective against vancomycin-resistant strains. Dimerization of these compounds by disulfide formation and olefin metathesis led to the identification of an additional set of highly potent antibiotics [33••]. In this case, the discovery of active compounds was facilitated through the use of target-accelerated combinatorial synthesis (or dynamic combinatorial synthesis) [34,35].

It has been suggested that glycolipid derivatives of vancomycin (i.e. compounds containing a lipid-functionalized disaccharide) are active against resistant strains of bacteria because of their ability to inhibit the transglycosylation step of peptidoglycan biosynthesis [36,37]. If this model is correct, it should be possible to improve the activity of vancomycin derivatives by optimizing the glycolipid moiety for inhibition of transglycosylation. In order to test this hypothesis, Kahne and co-workers [38] devised a strategy for the synthesis of a new class of vancomycin analogs, termed hybrid glycopeptide antibiotics. To illustrate their approach, the aglycone was modified by alkylation with a synthetic disaccharide, corresponding to an analog of the known transglycosylase inhibitor moenomycin (Figure 5b). This disaccharide had been identified from a combinatorial library of moenomycin analogs [39]. The resulting hybrid

Figure 5



Strategies for the synthesis of vancomycin analogs. (a) Nicolaou's synthesis of vancomycin analogs by reductive amination with benzaldehyde derivatives, containing terminal alkenes and thioacetates [31]. Dimerization of the vancomycin analogs (by disulfide formation or olefin metathesis) led to the identification of potent antibiotics with activity against resistant bacterial strains [33••]. (b) Kahne's hybrid glycopeptide antibiotic, containing a disaccharide analog of the transglycosylase inhibitor moenomycin [38].

molecule, which contains the vancomycin aglycone in place of the lipid moiety, exhibits antibiotic activity far exceeding that of the individual components. This approach should greatly facilitate the synthesis of a large collection of vancomycin analogs, because the synthetically challenging glycosidic linkage is replaced with a simple ethylene glycol linker.

Conclusions

In light of the biological importance of oligosaccharides [40], the development of new strategies for their preparation is key to the advancement of our understanding of various carbohydrate–protein interactions and the discovery of new therapeutic agents. The application of combinatorial synthesis to the production of carbohydrate-based libraries has received increased attention in recent years. Combinatorial strategies have been applied to the discovery of new carbohydrate-based antibiotics, including derivatives of vancomycin [31;33••,38] and aminoglycosides [29], and novel one-pot glycosylation strategies have been employed for the generation of oligosaccharide libraries [6••,7•]. Recent advances in solid-phase oligosaccharide synthesis, resulting in the development of an automated synthesizer [15••], are expected to facilitate future progress in the assembly of carbohydrate-based libraries.

Acknowledgements

LAM would like to thank the NIH for a post-doctoral fellowship.

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- •• of outstanding interest

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